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European J. Biochem. 9 (1969) 245—249

A New Method for the Extraction of R Lipopolysaccharides

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(Received February 4/March 21, 1969)

A method is described which is specific for the extraction of lipopolysaccharides from R form bacteria. The extraction mixture which is monophasic, consists of aqueous phenol, chloroform and petroleum ether. R form lipopolysaccharides (glycolipids), due to their lipophilic nature, are completely soluble in the mixture. S and T form lipopolysaccharides as well as proteins, nucleic acids, and polysaccharides, are insoluble, and they are excluded from the extracts. The method is mild, as it can be carried out at below 10°. The yields are generally higher than those obtained by phenol-water extraction, and the products are usually water-soluble. Lipopolysaccharides have been successfully extracted from all R form bacteria so far attempted.

Many methods have been developed for the extraction of endotoxic lipopolysaccharides from *Salmonella* and other gram-negative bacteria [1,2]. One of the methods, which is widely used, is the phenol-water procedure [3,4], because it is applicable to many different groups of bacteria and because it is one of the few methods by which also lipopolysaccharides from R mutant bacteria can be extracted. In this method, dried bacteria are treated at 69° in a mixture of phenol and water (45:55, v/v). On cooling, the mixture, which is monophasic above 68°, separates into a phenol phase containing mainly proteins, and a water phase containing lipopolysaccharide and nucleic acid.

However, there are recent reports in the literature describing unexpected results. Ashwell *et al.* [5] extracted *Xanthomonas campestris* bacteria with phenol—water and obtained a phenol-soluble lipopolysaccharide. Similarly, Raff and Wheat [6] applied the phenol—water procedure to *Citrobacter* strains and obtained the respective lipopolysaccharides from the phenol phase. Finally, Kasai and Nowotny [7], who investigated a *Salmonella* R mutant, isolated the corresponding heptose-less lipopolysaccharide (glycolipid) from the phenol interphase, though, in our hands, the phenol—water extraction of the same mutant yielded an identical lipopolysaccharide (glycolipid) in the water phase [8], however, in a rather low yield.

All the lipopolysaccharides found to be soluble in the phenol phase seem to have the common characteristic of being hydrophobic in nature. The lipopolysaccharides from *X. campestris* and *Citrobacter* contain 3-*N*-acetylamino-3,6-dideoxyhexose [5,9,10] and the heptose-less lipopolysaccharide of the R mutant contains besides 2-keto-3-deoxyoctonate (KDO) about 70% of lipid A [8].

Unusual Abbreviation. KDO, 2-keto-3-deoxyoctonate.

The present paper describes an extraction method which is specific for R form lipopolysaccharides which are obtained as water-soluble preparations of high purity regarding contamination with proteins and nucleic acid. S form lipopolysaccharides are excluded from the extract, and also certain polymers, like glucans, are not co-extracted.

MATERIALS AND METHODS

Bacteria

The following S and R strains were used: *S. minnesota* S form, mR345, mRz, mR595 [8], *S. godesberg* gR15 (isolated by J. Schlosshardt), and *S. typhimurium* TV 161 [11]. They were cultivated as described previously [12]. After harvesting, the bacteria were washed with distilled water. Saline or any other salt solution should be strictly avoided, as such a treatment may result in a very poor lipopolysaccharide yield. The washed bacteria are treated successively with ethanol, acetone and twice with ether. They are dried *in vacuo* over $CaCl_2$ to constant weight.

Extraction Mixture

A mixture containing liquid phenol (90 g dry phenol + 11 ml water), chloroform and petroleum ether (b. p. 40—60°) in a volume ratio of 2:5:8, respectively, was used. This mixture was a monophasic system if the phenol used was dry. If water was present in the original phenol preparation, the mixture was cloudy; it could be made clear by adding solid phenol.

Extraction Procedure

The dried bacteria (50 g) were placed in a centrifuge vessel and the extraction mixture (200 ml)

was added. The suspension was then homogenised with the Ultra-Turrax homogenisator (Janke & Kunkel) for 2 min with cooling so that the temperature remained between 5° and 20°. This treatment was not meant to break the bacteria but to obtain them in a fine suspension. If the bacteria were already in a fine form, stirring of the mixture for a few minutes was sufficient. Sometimes the suspension after homogenising was very viscous. In this case more extraction mixture was added. The bacteria were then centrifuged off (5000 rev./min, 15 min) and the supernatant which contained the lipopolysaccharide was filtered through filter paper into a round flask (1 l). The bacterial residue was extracted once more with the same amount of extraction mixture, stirred and centrifuged as above and the supernatant was added to the first extract. The extraction could be repeated for a third time.

The pooled supernatant solutions had a light yellow to dark brown colour. Petroleum ether and chloroform were then removed completely on a rotary evaporator at 30—40° (or in high vacuum at below 0°). Should the remaining phenol now crystallise, sufficient water was added to dissolve it. The solution was transferred into a glass centrifuge pot and water added dropwise until the lipopolysaccharide precipitated. Addition of water was stopped when the lipopolysaccharide started settling down after the mixture was allowed to stand for 1 to 2 min. Although precipitation of the lipopolysaccharide is complete long before the phenol is saturated with water, care must be taken not to add too much water as this causes formation of two phases. The precipitated lipopolysaccharide was then centrifuged (3000 rev./min, 10 min), the supernatant decanted, and the tube allowed to stand for 2 to 3 min upside down. It was then wiped inside with filter paper. The precipitate was washed two to three times with small portions of 80% phenol (about 5 ml) and the inside of the tube was wiped with filter paper after decantation of the supernatant. Finally, the precipitate was washed three times with ether to remove any remaining phenol, and dried *in vacuo*. The lipopolysaccharide was taken up in distilled water (50 ml), warmed to about 45°, and vacuum was carefully applied to remove the air. It was then shaken for a few minutes whereby a viscous, sometimes very viscous, solution was obtained. The viscosity may be reduced by placing the solution in an ultra vibrator for 5 min. The lipopolysaccharide solution was centrifuged once at high speed (100000 × *g*, 4 h). The resulting sediment was clear and transparent, so that it was sometimes difficult to recognise until the supernatant was decanted. The lipopolysaccharide was redissolved in water and freeze-dried.

The supernatant solution after ultracentrifugation contained negligible amounts of material and was discarded. It did not contain O-specific hapten [13].

RESULTS

Extraction of S and T_1 Form Bacteria

A number of S and T_1 lipopolysaccharides which had been extracted with phenol—water [3,4,8], when tested for their solubility in the phenol—chloroform—petroleum ether mixture, were found to be insoluble. When *S. typhimurium* S form and *S. friedenau* T_1 form bacteria were subjected to the new extraction method no material was found in the extract. Phenol—water, on the other hand, extracted from the same batches of bacteria the respective lipopolysaccharides in usual yields.

Extraction of R Form Bacteria and Analysis of the Respective Lipopolysaccharides

The new method of extraction with a mixture of phenol, chloroform and petroleum ether, as described in Methods, has been successfully applied to a large number of R mutant bacteria and, without exception, the respective lipopolysaccharides were obtained. In order to compare lipopolysaccharides extracted by phenol—water [3,4] with those obtained by the new method, one batch of each of 5 bacterial R mutants strains was split and each half was extracted separately by the two procedures respectively. The Table summarizes the results.

Table. *Comparative analysis of R lipopolysaccharides extracted by the phenol—chloroform—petroleum ether and phenol—water methods*
A = values for phenol—chloroform—petroleum ether extracts. B = values for phenol—water extracts. NT = Not tested. — = Not tested because of poor yield

Lipopolysaccharide		Yield	KDO	Heptose	Glucose	Galactose	Lipid A
		%	%	%	%	%	%
S. minnesota RZ	A	2.4	14.4	17.7	0	0	64
	B	0.88	15.0	15.4	0	0.5	52
S. minnesota R595	A	5.5	23.0	0	0	0	70
	B	0.6	21.0	0	0	0	65
S. godesberg R15	A	5.3	17.0	0	0	0	68
	B	0.1	—	—	—	—	—
S. minnesota R345	A	1.2	15.0	17.5	4.5	8.5	NT
	B	NT	12.0	14.0	4.0	9.0	NT
S. typhimurium TV 161	A	3.2	10.8	16.0	4.0	7.7	NT
	B	2.0	8.9	15.8	6.8	8.7	NT

In each case the yield obtained by the new method was significantly higher. From *S. godesberg* gR15 practically no lipopolysaccharide was obtained with phenol—water, but more than 5% by the new method (see the Table). Sugar analyses performed on the respective pairs of lipopolysaccharides showed that they contained the same sugar constituents, in about the same quantities.

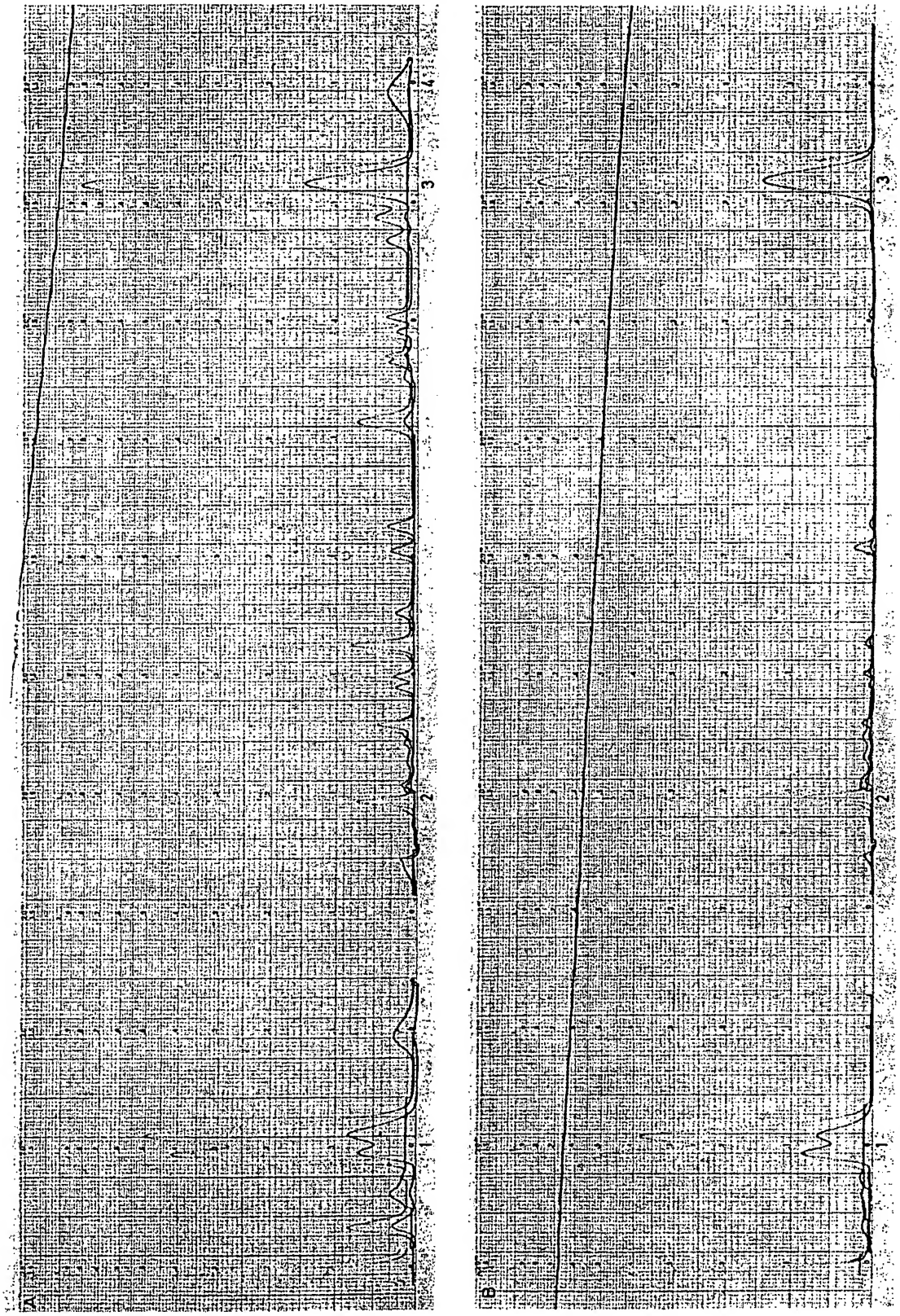

Fig. 1. *Amino acid spectra of the lipopolysaccharides of* S. minnesota *mRe obtained by the phenol-water method (A) and by the phenol-chloroform-petroleum ether method (B).* 1 ethanolamine; 2 ethanolamine phosphate, glucosamine phosphate; 3 glucosamine; 4 galactosamine

Nucleic acid was absent from all lipopolysaccharides. The protein (or peptide) content in each of the lipopolysaccharides obtained from the mutant mRz by the respective procedure was compared in the amino acid analyzer after hydrolysis of the extracts in 6 N HCl for 24 h (Fig. 1 A and 1 B). A number of amino acid peaks are seen on Fig. 1 A (phenol—water extraction), corresponding to a 0.1 to 1 % total in the preparation. This amount is significantly reduced in the phenol—chloroform—petroleum ether extracted lipopolysaccharide (Fig. 1 B). Peaks of ethanolamine and glucosamine are seen in both lipopolysaccharides. The phenol—water extracted preparations shows the presence of small amounts of galactosamine, indicating contamination with S form lipopolysaccharide which is synthesized by this mutant mRz due to a leaky defect [14]. This S form contaminant is absent from the phenol—chloroform—petroleum ether extracted preparation, a conclusion derived from the fact that the galactosamine peak is absent. This is also supported by the absence of galactose.

Sequential Extraction of S. minnesota *mR60 Bacteria*

On extraction of *S. minnesota* mR60 bacteria with the phenol—chloroform—petroleum ether mixture, a lipopolysaccharide (1 % yield) was obtained. Sugar analyses revealed the presence of KDO (12 %), heptose (17 %), glucose (6 %) and galactose (6 %). No ribose was detectable. The bacterial residues of the first extraction were subsequently extracted with phenol—water and a second lipopolysaccharide was obtained (0.7 % yield) containing KDO (9 %), heptose (13 %), glucose (7 %), galactose (10 %), and in addition, ribose (10 %). It is concluded, that the mutant mR60 synthesises two lipopolysaccharides, one being a typical R lipopolysaccharide, the other being a T_1-like lipopolysaccharide (see also [8]).

Isolation of O-Specific Hapten

When cells of *S. typhimurium* TV161 are extracted with phenol—water and the extract is subjected to high speed centrifugation, the corresponding R lipopolysaccharide is obtained as a sediment (2 % yield, see Table 1), while the supernatant solution contains O-specific hapten [13].

Extraction of the same cells by the phenol—chloroform—petroleum ether method yields R lipopolysaccharide (3.2 % yield, Table). No O-specific hapten is found in the extract. The cell residue after this extraction was treated with phenol—water. The water-phase obtained yielded, on high speed centrifugation, a small amount of sediment (0.4 %) and a supernatant containing the O-specific hapten. Its presence was demonstrated after hydrolysis by paper chromatography: glucose, galactose, mannose, rhamnose and abequose were detected [13].

DISCUSSION

An important factor in the phenol—water method for the extraction of bacteria is the distribution of lipopolysaccharide between (water-saturated) phenol and (phenol-saturated) water. Because of their relative hydrophilic character, lipopolysaccharides from enterobacterial S (wild type) forms are generally excluded from the phenol phase and they are found almost exclusively in the water phase. However, in the case of more lipophilic lipopolysaccharides, such as those derived from R mutants, a different distribution between the two phases could be expected, and here, several, otherwise not so critical factors could govern the yield, in which such lipopolysaccharides are obtained from the water or phenol phase, respectively. One could imagine, for instance, that the kind and/or quantity of cations, or small differences in the temperature to which the phenol—water mixture is cooled, might influence the distribution of the more lipophilic lipopolysaccharides, and it is not surprising on the basis of these considerations that reports have been published where a lipopolysaccharide was found, after extraction, in the water phase, in the phenol phase or at the interphase ([7], see also [5,6]) of a phenol—water system. The lipopolysaccharide in question was derived from a *Salmonella* R mutant and is known to be highly lipophilic since it contains only KDO and about 70 % of lipid A [8]. The same is true for R mutant lipopolysaccharides in general, which are devoid of the long O-specific side chains which confer hydrophilic properties to the S form lipopolysaccharides. Regarding the S form lipopolysaccharides which have been isolated from the phenol or interphase [5,6] it is assumed that they are relatively lipophilic due to the presence of deoxy- and deoxy-*N*-acetylamino-hexoses.

The present paper describes an extraction method, where aqueous phenol, chloroform, and petroleum ether is used as extraction mixture, which has been applied successfully to *Salmonella* R mutants of all R classes (Ra to Re [8]) for the isolation of the respective R lipopolysaccharides. No S or T_1 lipopolysaccharides are extracted from either S or T_1 form bacteria, respectively.

The R lipopolysaccharides which were obtained by the new method in higher yields, compared with the phenol—water method, proved to be pure with respect to contaminants such as protein, nucleic acids and polymers like glucan. The S-form lipopolysaccharide present in a leaky mutant (mRz) was completely excluded from the new extract. Also, the O-specific hapten present in Rb class mutants is not co-extracted with the Rb lipopolysaccharide; it can be obtained when the cell residues are subsequently extracted with phenol—water. When cells of the R mutant strain of *S. minnesota* mR60 [8] were extracted with phenol—chloroform—petroleum ether, a R lipopolysaccharide was obtained. A second (T_1-like) lipo-

polysaccharide [8] containing ribose could be extracted by further extraction of the cell residues with phenol—water.

The new method of extraction can be applied to small amounts of bacteria. It is relatively mild, because it is carried out at temperatures between 5° and 20°. It is also time-saving, because the long dialysing periods and the three-fold ultra-centrifugation of the phenol—water method are not necessary in this procedure. The R lipopolysaccharides obtained are usually completely soluble in water. Addition of salt, such as $MgCl_2$ or $CaCl_2$ (or larger amounts of NaCl) precipitates these lipopolysaccharides from their solution. This may be a reason why lipopolysaccharides are not extractable from bacteria that had been washed with salt solutions during harvesting.

The authors are indebted to Fräulein I. Minner and Fräulein H. Winkler for expert technical assistance, and to Herr R. Warth for carrying out the amino acid analysis.

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